ELSEVIER

Gene 255 (2000) 297–305

GENE
AN INTERNATIONAL JOURNAL ON GENES, GENOMES AND EVOLUTION

www.elsevier.com/locate/gene

Regulated gene expression in *Staphylococcus aureus* for identifying conditional lethal phenotypes and antibiotic mode of action

Ling Zhang, Frank Fan *, Leslie M. Palmer, Michael A. Lonetto, Chantal Petit, LeRoy L. Voelker, Annemarie St. John, Brent Bankosky, Martin Rosenberg, Damien McDevitt

Anti-Infectives Research, SmithKline Beecham Pharmaceuticals Research and Development, 1250 S. Collegeville Road, Collegeville, PA 19426, USA

Received 23 May 2000; accepted 17 July 2000
Received by D.L. Court

Abstract

Selectively regulating gene expression in bacteria has provided an important tool for studying gene function. However, well-regulated gene control systems have been restricted primarily for use in laboratory non-pathogenic strains of bacteria (e.g. *Escherichia coli*, *Bacillus subtilis*). The development of analogous systems for use in bacterial pathogens such as *Staphylococcus aureus* would significantly enhance our ability to examine the contribution of any given gene product to pathogen growth and viability. In this report, we adapt, examine and compare three regulated gene expression systems in *S. aureus*, which had previously been used in *B. subtilis*. We demonstrate that all three systems function and exhibit titratable induction, together covering a dynamic range of gene expression of ~3000-fold. This dynamic range correlates well with the physiological expression levels of cellular proteins. Importantly, we show that one of these systems, the Spac system, is particularly useful for examining gene essentiality and creating specific conditional lethal phenotypes. Moreover, we find that titration of selective target gene products using this system allows direct demonstration of antibiotic mode of action.

Keywords: Methionyl tRNA synthetase; Polypeptide deformylase; Regulated promoter; Spac; Xyl; Xyl/tet

1. Introduction

The selective modulation of gene expression levels is a very powerful strategy in determining the contribution of a given gene product to bacterial growth and/or pathogenesis. Valuable information is gained by modulating gene expression levels and monitoring the effect as the product titrates up or down. Compared with traditional gene inactivation/replacement strategies, which typically result in the total loss of gene function, regulated gene expression can provide quantitative information about the functional importance of a gene product. It also allows the creation of conditional lethal phenotypes when genes are essential. Such information is useful for studying gene function and evaluating molecular targets for antibiotic discovery.

An ideal regulated gene expression system should enable the modulation of gene expression over a wide range of levels starting from no expression. In Gram-negative laboratory strains of bacteria such as *Escherichia coli*, a variety of regulated promoter systems including P_{BAD}, P_{lac}, P_{tac}, P_{trc}, P_L and P_R, and P_{T7} (see Guzman et al., 1995 and references cited therein) have been developed. Some regulated promoter systems have also been developed for use primarily in non-pathogenic Gram-positive bacteria. These include the P_{spac}, P_{xyl} and $P_{xyl/tet}$ systems in *Bacillus* species (Yansura and Henner, 1984; Kim et al., 1996; Geissendorfer and Hillen, 1990), and the P_{nisA} and P_{lacA} systems in *Lactococcus lactis* (Eichenbaum et al., 1998). More recently, a P_{tet} system was described in *Streptococcus pneumoniae* (Stieger et al., 1999).

Abbreviations: ATc, anhydrotetracycline; βGal, β-galactosidase; bp, base pairs; IPTG, isopropyl-β-D-thiogalactoside; kb, 1000 bp; LB, Luria–Bertani; MCS, multiple cloning sites; MetRS, methionyl tRNA synthetase; MIC, minimum inhibitory concentration; OD_{600}, optical density at 600 nm; *P*, promoter; PCR, polymerase chain reaction; Pdf, polypeptide deformylase; SDS, sodium dodecyl sulfate; Spac, P_{spac} and *lacI* regulon; TSA, tryptic soy agar; TSB, tryptic soy broth; Xyl, P_{xyl} and *xylR* regulon; Xyl/tet, $P_{xyl/tet}$ and *tetR* regulon; ::, novel junction (fusion).

* Corresponding author. Tel.: +1-610-917-6674; fax: +1-610-917-4989.
E-mail address: frank_fan-1@sbphrd.com (F. Fan)

0378-1119/00/$ - see front matter
PII: S0378-1119(00)00325-5

The Gram-positive bacterium *Staphylococcus aureus* is a versatile pathogen responsible for a variety of pyogenic and toxin-related infections. Its pathogenesis is complex and involves the coordinated expression of multiple virulence genes in response to changes in the bacterial environment. Significant progress has been made in studying the biology and pathogenesis of this organism with the development of molecular tools such as plasmid vectors, transducing phages, transposons and reporter genes (Novick, 1991). Although regulated expression has been reported for antiseptic resistance, antibiotic resistance and sugar operons in *S. aureus* (Ji and Silver, 1992; Gregory et al., 1997; Oskouian and Stewart, 1987), only the β-lactamase inducible system has been adapted for use in genetic studies (Iordanescu, 1993). Recently, we reported the adaptation of the tetracycline-inducible Xyl/tet ($P_{xyl/tet}$ and *tetR* regulon) system to induce the expression of antisense RNA to down-regulate the expression of the *S. aureus* α-toxin gene (Ji et al., 1999).

In this report, we compare in *S. aureus* the expression properties of the Xyl/tet system with two other regulated promoter systems (P_{xyl} and P_{spac}) that had been developed previously for use in *Bacillus*. The tetracycline-inducible Xyl/tet hybrid system consists of the *Bacillus subtilis*-derived P_{xyl} promoter and the *E. coli*-derived *tet* regulatory elements from Tn*10* (Geissendorfer and Hillen, 1990). The xylose-inducible Xyl (P_{xyl} and *xylR* regulon) system consists of the *Bacillus megaterium* P_{xyl} *promoter and XylR repressor of the xylose utilization operon (Kim et al., 1996). The isopropyl-β-*D*-thiogalactoside (IPTG)-inducible Spac (*P_{spac} *and lacI* regulon) system is another chimeric promoter consisting of the *E. coli lac* operator fused to a promoter from the *B. subtilis* phage SPO-1 (Yansura and Henner, 1984). The repressor gene for the Spac system, *E. coli lacI*, is under the control of a constitutive promoter of a penicillinase gene (P_{pcn}) from *Bacillus licheniformis* (Yansura and Henner, 1984). Our results demonstrate that each of these promoter systems functions in *S. aureus*, and spans overlapping ranges of transcription control levels. We focus on the utility of the Spac system for determining gene essentiality since it can be titrated down to very low basal levels of expression. In addition, we demonstrate how modulating target expression levels (using the Spac system) can be a powerful strategy in linking the antibacterial activity of selected compounds with their proposed cellular target in a major pathogen *S. aureus*.

2. Materials and methods

2.1. Bacterial strains and culture conditions

E. coli DH5α was the host for all plasmid constructions and maintenance. *S. aureus* strains RN4220 (Kreiswirth et al., 1983) and OS2 (Schneewind et al., 1992) were used as hosts. *E. coli* strains were grown in Luria–Bertani broth (LB; BBL, Sparks, MD) and 100 μg/ml of ampicillin was used for selection. *S. aureus* strains were grown in LB or tryptic soy broth (TSB; BBL), or on tryptic soy agar (TSA). For selection, 75 μg/ml of kanamycin or 5 μg/ml of erythromycin was used.

2.2. Plasmid construction

Xyl/tet, Xyl and Spac were PCR amplified from pWH353 (Geissendorfer and Hillen, 1990), pX (Kim et al., 1996) and pMUTIN4 (Vagner et al., 1998), respectively using the following primer sets: Xyl/tet, 5′-TTTAAAATCGATAACTCGACATC-3′ and 5′-AGATCTGATATCAAGCTTATTTTAA-3′; Xyl, 5′-GCATGCTAACCTTTGCGTTCACTTAACTAA-3′ and 5′-GGATCCTGCAGCCATGGATTTCCCCCTTTGATTTTTAGATATCAC-3′; and Spac, 5′-TTTAAATCGGATCTGGTAATGACTCT-3′ and 5′-CAATTGTCACTGCCCGCTTTCCAGTC-3′. Each PCR fragment was cloned into the multiple cloning sites (MCS) of an *E.coli–S. aureus* shuttle vector pRB373 (Bruckner, 1992), yielding plasmid vectors pLZ113, pLZ97 and pLZ109, respectively (Fig. 1).

A modified version of the *E. coli lacZ* gene encoding β-galactosidase (βGal), that had previously been used successfully as a reporter gene in *B. subtilis* and *S. aureus* (Vagner et al., 1998; Chan and Foster, 1998), was PCR amplified from pMUTIN4 and cloned into the MCS region in pLZ113, pLZ97 and pLZ109. The resulting shuttle plasmids carrying regulated promoter–*lacZ* fusions were named pLZ114, pFF52 and pLZ119, respectively.

The PCR fragment amplified from pMUTIN4 for construction of pLZ109 was also digested with *Hin*dIII and *Mfe*I and then ligated with *Hin*dIII–*Eco*RI digested pRB373. The resulting plasmid is called pFF40. It contains the *lacI* gene under the control of the P_{pcn} promoter from *B. licheniformis*.

2.3. Construction of S. aureus strains

FFmrs1 (*metRS*::P_{spac}), primers 5′-ATGCGAATTCAGACATTACGAGGAGGAACAG-3′ and 5′-ATGCGGATCCTTAAACTTCGTGTCCAGAATCTGG-3′ (containing *Eco*RI and *Bam*HI sites, respectively) were used to PCR amplify, from *S. aureus* RN4220 genomic DNA, a 0.5 kb fragment from the 5′ end of *metRS*, including a putative ribosome binding site. The PCR fragment was digested with *Eco*RI and *Bam*HI and cloned into pSMUTery integration vector (a derivative of pMUTIN4 without the *lacZ* gene, a gift from Simon Foster, University of Sheffield, UK). The resulting integration plasmid pFF81 was transformed into *S. aureus*

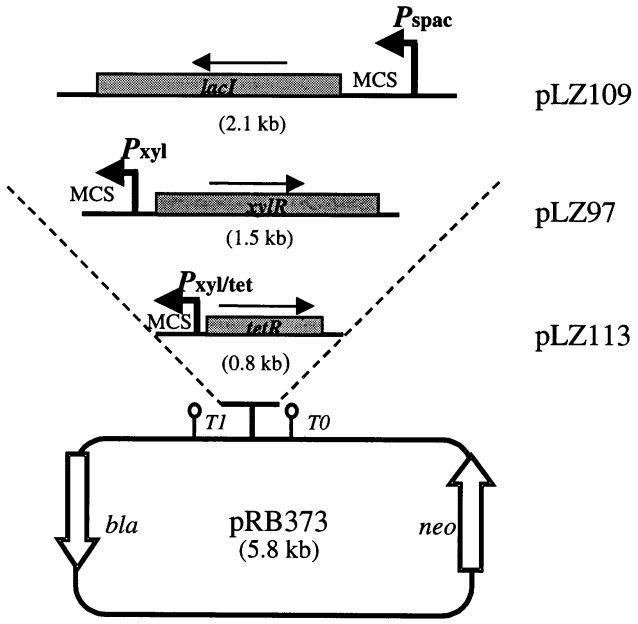


Fig. 1. Diagrammatic representation of shuttle plasmids pLZ113 (Xyl/tet), pLZ97 (Xyl) and pLZ109 (Spac). Each of these plasmids is derived from pRB373, with the insertion of different promoters and their cognate regulator genes. The unique restriction sites in the MCS region of each shuttle plasmid are: pLZ109, 5′-*Eco*RI, *Sma*I, *Not*I, *Sac*II, *Bam*HI, *Sph*I, *Nsp*V, *Sac*I-3′; pLZ97, 5′-*Pst*I, *Bam*HI, *Sma*I, *Kpn*I, *Sac*I-3′; pLZ113, 5′-*Hin*dIII, *Eco*RV, *Sma*I, *Kpn*I, *Sac*I, *Eco*RI-3′. *T1* and *T0* are terminators from *E. coli rrnB* and phage λ, respectively. *bla* encodes an ampicillin resistance determinant for selection in Gram-negative bacteria and *neo* encodes a neomycin/kanamycin resistance determinant for selection in Gram-positive bacteria. Drawing is not to scale.

RN4220 by electroporation following a procedure described by Kraus and Iandolo (1990). The single crossover insertion-generated strain FFmrs1 was resistant to erythromycin as expected.

Strain FFdef1 (*def1*::P_{spac}) was generated using the same method as described above. Primers for PCR amplification of the truncated *def1* gene are: 5′-ATGCGAATTCAGGAAGGTGCAATATATGTTA-AC-3′ and 5′-ATGCGGATCCTTAGAAAACAATT-GCTGGATATCC-3′.

2.4. βGal activity assay

S. aureus strains were grown in LB broth with appropriate antibiotics and monitored by measuring optical density at 600 nm (OD_{600}). Inducers were added to the culture when the OD_{600} reached ~0.5. Cell pellet from 500 μl of culture was collected by centrifugation 3 h after induction and stored at −20°C until assayed. βGal activity was determined as described by Nicholson and Setlow (1990), except that lysostaphin (100 μg/ml, AMBI Inc., Purchase, New York) was used to lyse the cells at 37°C for 10 min.

2.5. TaqMan quantitative RT-PCR

S. aureus cell samples were collected as described for the βGal activity assay, snap-frozen in a dry ice/ethanol bath and stored in liquid nitrogen until processed. Total RNA was isolated and reverse transcribed to cDNA using commercial kits following the manufacturers' instructions (FastRNA-Blue Kit from BIO101, Vista, CA; MessageClean Kit from GeneHunter, Nashville, TN; SuperScript Preamplification System for First Strand cDNA Synthesis Kit from Life Technologies, Gaithersburg, MD).

PCR reactions were set up using the TaqMan PCR Core Reagent Kit (PE Applied Biosystem, Foster City, CA) according to the instructions provided. Real-time sequence-specific detection and relative quantitation were achieved with the ABI PE Sequence Detection System 7700 (Heid et al., 1996; Holland et al., 1991). Relative amounts of *lacZ* cDNA were normalized to *S. aureus* 16S rRNA as an endogenous control. The amount of *lacZ*, normalized to 16S rRNA and relative to a calibrator (wild-type cells), was approximated by $2^{-\Delta\Delta C_T}$. The ΔC_T value, or difference in threshold cycle between target and control, was determined by subtracting the average 16S rRNA C_T value from the average *lacZ* C_T value. $\Delta\Delta C_T$ involved subtraction of the resultant value from the ΔC_T value of the calibrator. Forward and reverse primers (5′-AGCGGCATTTTCCGT-GAC-3′ and 5′-GGCAACATGGAAATCGCTG-3′, respectively) were designed to amplify a 69 bp fragment of the *E. coli lacZ* reporter cDNA transcribed from the shuttle vectors. For the endogenous control, forward and reverse primers (5′-CGTCACACCACGAGAGT-TTGTAA-3′ and 5′-ACCTTTCGACGGCTAGCT-CC-3′, respectively) were designed to amplify a 70 bp fragment at the 3′-end of the *S. aureus* 16S rRNA. The corresponding probes, 5′-[FAM]-TCTCGTTGCTGC-ATAAACCGACTACACAAA-[TET]-3′ and 5′-[FAM]-CCCGAAGCCGGTGGAGTAACCTTTT-[TET]-3′, complementary to *E. coli lacZ* and *S. aureus* 16S rRNA, respectively, were obtained from PE Applied BioSystems.

2.6. Western immunoblotting and estimation of protein copy number

S. aureus cell samples were collected and lysed as described in the βGal activity assay. Samples were heated at 95°C in Protein Sample Buffer (100 mM Tris–HCl, pH 8, 2% SDS, 0.1% Bromphenol Blue, 10% glycerol) for 10 min. SDS polyacrylamide gel electrophoresis and transfer of protein from gel to polyvinylidene difluoride (PVDF) membrane were carried out using the NuPAGE Electrophoresis System and following the manufacturer's instructions (NOVEX, San Diego, CA). Western immunoblotting was performed

according to the protocol supplied with the ECL Plus Detection Kit (Amersham, Cleveland, OH). The signal was recorded on Hyperfilm ECL (Amersham, Cleveland, OH) or a PhosphoImager (Molecular Dynamics, Sunnyvale, CA). Purified protein of interest was used as standard and run on the same gel. The quantity of the protein of interest in the whole-cell sample was determined compared with the standards.

A separate sample of the culture (0.5 ml) was sonicated briefly at 10% output for 15 s using a sonicator with microtip (Sonics and Materials, Inc., Danbury, CT). This treatment effectively breaks up cell clumps into single cells without lysing the cells. The cell density of this culture was determined by plate count on TSA media as well as by flow cytometry (Bryte-HS flow cytometer, Bio-Rad Laboratories, Hercules, CA). The protein copy number per cell was estimated using the following formula: [protein amount (gram/molecular weight) × Avogadro's number]/cells in the sample.

2.7. Growth curves of S. aureus strains

S. aureus cell growth curves were collected using an automated microtiter plate format, which provides better reproducibility and higher throughput. *S. aureus* strains were grown at 37°C overnight in TSB with the appropriate antibiotics and 1 mM of IPTG. The cultures were diluted with fresh media containing the appropriate antibiotics to a final OD_{600} of about 1×10^{-4} ($\sim 5 \times 10^4$ cells/ml) and used as a starting culture. 198 μl of the starting culture was placed in a well of a flat-bottomed microtiter plate and mixed with 2 μl of IPTG stock solution to make a final IPTG concentration of 0, 2.5, 5, 10, 25, 50, 100, and 1000 μM. The cell growth at 37°C was monitored by measuring OD_{600} every 15 min with 1 min mixing before each reading, using a microtiter plate reader SpectraMax250 from Molecular Devices (Sunnyvale, CA).

3. Results and discussion

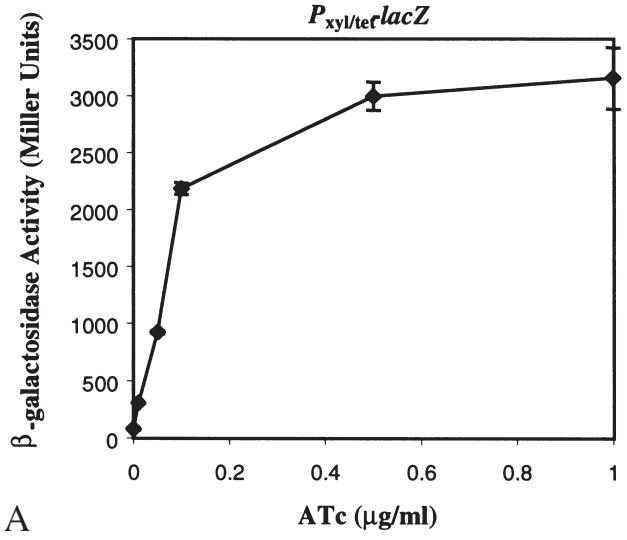
3.1. Evaluation of the Xyl/tet, Xyl and Spac expression systems in S. aureus

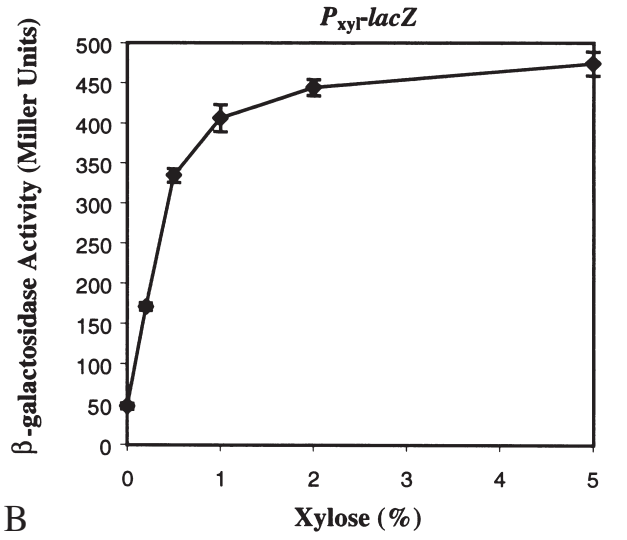
Expression from the different regulated promoter systems was compared using the same plasmid vector and reporter gene. We used an *E. coli*/*S. aureus* shuttle plasmid pRB373 (Bruckner, 1992) as vector and *E. coli lacZ* as reporter gene. Each plasmid with promoter–*lacZ* fusion was transformed into *S. aureus* strain OS2 and the resulting strains were examined for their ability to express βGal when induced.

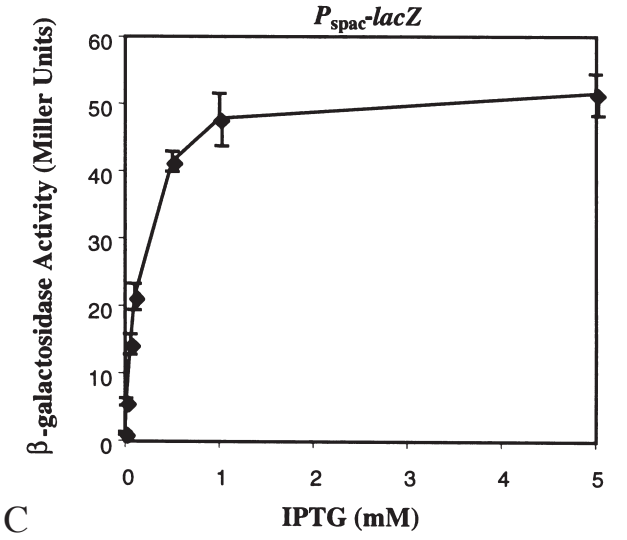
Experiments examining the time course of induction indicated that steady-state *lacZ* gene expression reached its maximum within 3 h after induction for each of the systems (data not shown). Therefore, all subsequent inductions were analyzed 3 h following the addition of inducer. Our results demonstrated that all three regulatory systems showed efficient and titratable induction of βGal expression (Fig. 2). For strains with pLZ114 ($P_{xyl/tet}$–*lacZ*), pFF52 (P_{xyl}–*lacZ*) and pLZ119 (P_{spac}–lacZ), maximal specific activity was achieved with 0.5 μg/ml anhydrotetracycline (ATc), 2% xylose and 1 mM IPTG, respectively. When fully induced, the Xyl/tet system expressed the highest level of βGal activity (3100 units), which is about six-fold higher than from the Xyl system and 65-fold higher than from the Spac system. When uninduced, the Xyl/tet system also had the highest basal level of expression (90 units), twice that of the Xyl system and 90-fold higher than that of the Spac system. The basal level of expression from the Spac system (about 1 unit) was close to the background level of the host strain. Thus, the three systems cover a dynamic range of over 3000-fold of βGal expression from the basal expression level of the Spac system (1 unit) to the fully induced expression level of the Xyl/tet system (3100 units). The induction ratios (fully induced activity/uninduced basal activity) were about 30-fold for the Xyl/tet system, 10-fold for the Xyl system and at least 40-fold for the Spac system.

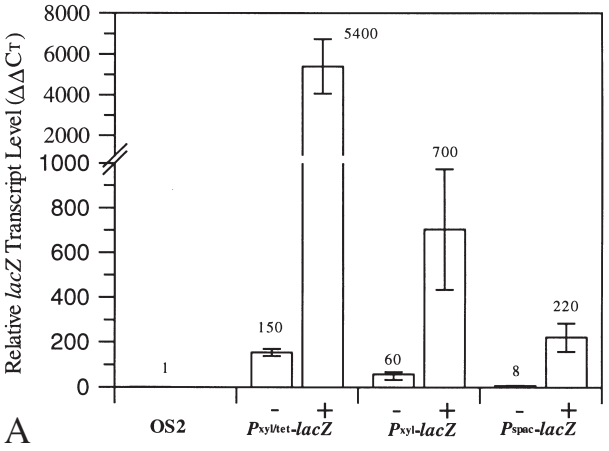
We then examined the steady-state mRNA transcript levels of the *lacZ* gene produced in the uninduced and fully induced states from each promoter system by TaqMan quantitative RT-PCR analysis. The results are shown in Fig. 3A. When fully induced, the Xyl/tet system showed the highest *lacZ* transcript level, about eight- and 25-fold higher than the Xyl and Spac systems, respectively. The uninduced basal level of *lacZ* transcript from the Xyl/tet system was about two-fold higher than from the Xyl system and 20-fold higher than from the Spac system. The induction ratios were 36-, 12- and 28-fold for the Xyl/tet, Xyl and Spac systems, respectively. These values are in general agreement with those obtained using the βGal enzyme assay, and demonstrate that the regulation of gene expression occurs primarily at the transcriptional level.

Finally, we examined the protein levels of βGal in uninduced and fully induced samples from each promoter system by Western immunoblotting analysis using an anti-βGal antibody. The results (Fig. 3B) were consistent with those obtained from both the enzyme activity and transcript level analysis, and allowed us to establish the average amount of βGal produced per cell by each system (see Section 2.6). The Spac system uninduced exhibited low/undetectable levels of βGal (lane 2) and when induced produced about 3 ng of βGal in a total of 7.5×10^6 cells, equivalent to $\sim 2 \times 10^3$ molecules per cell (lane 3). In contrast, the Xyl and Xyl/tet systems produced $3–5 \times 10^3$ molecules per cell when uninduced (lanes 4 and 6) and approximately $10^4–10^5$ molecules per cell when fully induced (lanes 5 and 7). The Xyl









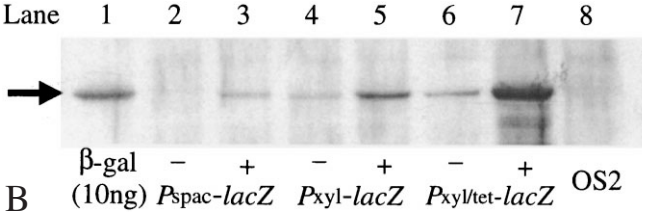


Fig. 3. TaqMan RT-PCR and Western immunoblotting analysis. The concentrations of the inducers were 0.5 μg/ml ATc, 2% xylose and 1 mM IPTG for the Xyl/tet, Xyl and Spac systems, respectively. Uninduced (−) and fully induced (+) samples were collected and assayed as described in Sections 2.5 and 2.6. (**A**) TaqMan RT-PCR analysis of *lacZ* mRNA. Strains from left to right, parent OS2; OS2(pLZ114); OS2(pFF52); and OS2(pLZ119). The averages of three measurements are marked on top of each column with standard deviations indicated by error bars. (**B**) Western immunoblotting using an anti-βGal antibody. Lane 1, βGal protein standard at 10 ng. Lanes 2 and 3, OS2(pLZ119). Lanes 4 and 5, OS2(pFF52). Lanes 6 and 7, OS2 (pLZ114). Lane 8, parent OS2. Each lane, except lane 1, was loaded with approximately 7.5×10^6 cells. Polyclonal rabbit anti-βGal antibody (5′–3′, Inc., Boulder, CO) and HRP-conjugated goat anti-rabbit IgG (Sigma, St. Louis, MO) were used at 1:500 and 1:2000 dilutions, respectively.

and Xyl/tet systems are 'leaky', but allow high levels of expression when fully activated. Thus, they are particularly useful for overexpression of gene products. In fact, recently the Xyl/tet system was used successfully to produce sufficient antisense RNA to down-regulate gene

Fig. 2. Titratable induction of the three regulated promoter systems in *S. aureus* using *E. coli lacZ* as a reporter gene. βGal activity was measured as described in Section 2.4. (**A**) OS2(pLZ114), $P_{xyl/tet}$–*lacZ*, ATc concentrations tested were: 0, 0.01, 0.05, 0.1, 0.5 and 1 μg/ml. (**B**) OS2(pLZ52), P_{xyl}–*lacZ*, xylose concentrations tested were: 0, 0.2, 0.5, 1, 2 and 5%. (**C**) OS2(pLZ119), P_{spac}–*lacZ*, IPTG concentrations tested were: 0, 0.01, 0.05, 0.1, 0.5, 1 and 5 mM. Each point and error bar represents the average of three βGal activity measurements and the standard deviation.

expression (Ji et al., 1999). In contrast, the Spac system is 'tight' and can produce, upon induction, reasonable levels of expression from a low basal level. This system is most appropriate for titrating the expression of gene products down from near physiological levels of a few thousand molecules per cell.

3.2. Gene essentiality testing using Spac

One application of regulating gene expression is to examine the requirement of any gene product for cell viability by titrating down its expression level and observing the consequences. In order to achieve this, we chose a strategy where the native promoter for any selected gene can be replaced by a single copy of the Spac promoter system in a homologous recombination event occurring on the *S. aureus* chromosome. We applied this method to the *S. aureus* gene *metRS* (GenBank accession No. A79563), which encodes methionyl tRNA synthetase (MetRS) (see Section 2.3 and Fig. 4A). The resulting strain (FFmrs1) carrying the appropriate insertion mutant was isolated and shown by Southern hybridization analysis (Fig. 4B) to contain the Spac promoter inserted immediately upstream of the *metRS* coding region. As a result of the insertion, this strain also carries a truncated *metRS* gene encoding the N-terminal 162 amino acid residues under the control of its native promoter (see Fig. 4A). This truncated *metRS* gene does not produce an active synthetase.

Growth of strain FFmrs1 was examined in liquid medium containing varying amounts of inducer IPTG. The results (shown in Fig. 5A) indicate that cell growth was only partially dependent on the extent of induction. Cultures containing ≥ 5 μM IPTG grew similar to wild-type with a doubling time of ~ 1 h. Cultures containing no IPTG grew significantly slower (doubling time 2.5 h) and achieved lower cell densities. However, even in the absence of inducer, growth was observed. We also examined growth of strain FFmrs1 on solid medium with or without inducer. In the absence of IPTG, colony size was reduced significantly, confirming that we had achieved a partial growth dependence phenotype (data not shown).

The *metRS* gene is essential for in vitro growth of *S. aureus* (unpublished data). Hence, the inability to achieve cell death for mutant strain FFmrs1 in the absence of inducer suggested that the uninduced basal expression from the P_{spac} promoter, when inserted into the chromosome, was producing enough MetRS to enable limited cell growth. Since the *lacI* repressor gene was also present in single copy, it was possible that there was insufficient LacI repressor being expressed. In order to test this idea, we decided to provide additional repressor and monitor the effect on P_{spac} expression. We constructed a plasmid, pFF40, with *lacI* expressed from the P_{pcn} promoter, and introduced pFF40 into strain FFmrs1. Again, cell growth was examined in response to IPTG inducer concentration. Cell growth now showed complete dependence on the level of inducer (Fig. 5B). No cell growth was observed in the absence of inducer when tested either in liquid (Fig. 5B) or on solid media (data not shown). Thus, by supplying sufficient repressor, Spac-regulated gene expression could effectively be used to create a conditional null *metRS* phenotype in *S. aureus*.

Creating conditional null phenotypes using regulated gene expression systems has been restricted primarily to non-pathogens such as *E. coli* and *B. subtilis* (Guzman et al., 1995; Petit et al., 1998). Recently, the *xyl* regulon from *Staphylococcus xylosus*, which is similar to that from *B. subtilis* tested in this study, was adapted for use in *S. aureus* to examine the requirement of *fmhB* gene product for cell viability (Rohrer et al., 1999). However, the *xylA* promoter was 'leaky' and only a slight decrease in growth rate was observed in the presence of the repressor. Similarly, a tetracycline-regulated system used in *S. pneumoniae* also appeared to be 'leaky' when the requirement of undecaprenyl pyrophosphate synthetase was tested for cell viability (Apfel et al., 1999). Apparently, high basal-level expression has been the major obstacle to using regulated expression systems for gene essentiality testing in pathogens.

We have demonstrated that the chromosomally-integrated Spac system, in combination with the LacI-expressing plasmid pFF40, is suitable for creating tightly regulated conditional null mutants in *S. aureus*. In addition to *metRS*, this strategy has now been applied successfully to a number of other genes (see another example below). This integration method allows easy placement of the Spac system upstream of any gene to yield IPTG-regulated expression. In cases where the gene of interest is part of an operon, the P_{spac} will potentially co-regulate downstream genes in that operon as well. In addition, this method requires the use of a truncated gene fragment of sufficient size (e.g. ~ 500 bp) to achieve efficient integration. Therefore, it may prove difficult to apply the technique to small genes. These limitations need to be kept in mind when using this system and interpreting the results.

3.3. Examination of antibiotic mode of action using Spac

Another important utility for regulated gene expression is to demonstrate that the cellular activity of an antibiotic is occurring through its proposed molecular target. By titrating the expression of the target gene up or down, it should be possible to shift correspondingly the measured susceptibility of the cell (i.e. MIC) to an antibiotic that works via the target. We describe an example of this application below.

Polypeptide deformylase (Pdf) is an essential enzyme that removes the N-formyl group from newly synthe-

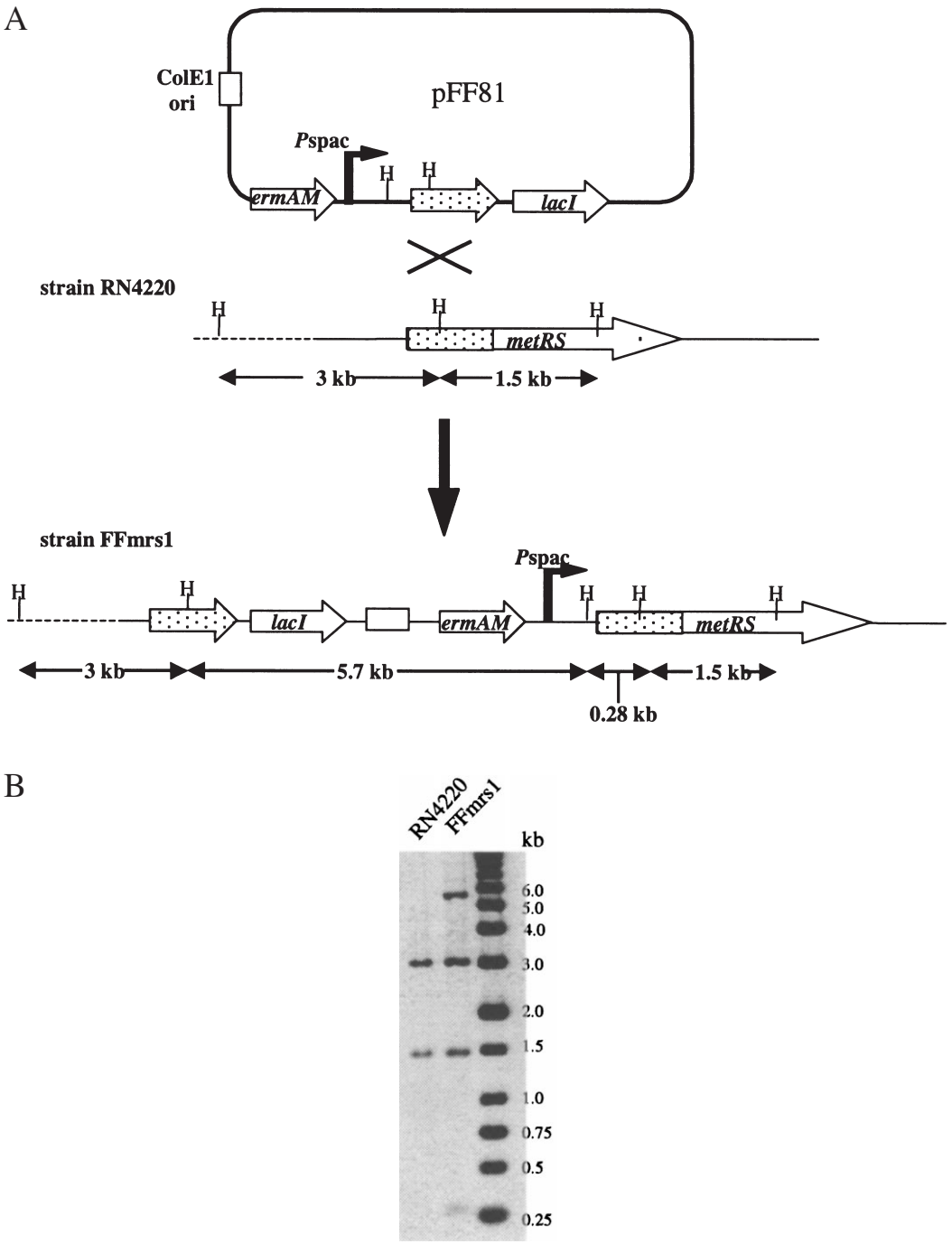


Fig. 4. Construction of an *S. aureus* strain, FFmrs1, with *metRS* under the control of P_{spac}. (**A**) An illustration of integration of pFF81 into the *S. aureus* RN4220 chromosome to yield strain FFmrs1 (not to scale, see Section 2.3). (**B**) Southern hybridization of *S. aureus* RN4220 and FFmrs1. The genomic DNA was digested with *Hin*dIII and probed with a fluorescein-labeled 0.5 kb truncated *metRS* gene (shaded box). The labeling and detection were performed according to the protocol supplied with ECL random prime labeling system version II (Amersham, Cleveland, OH). The sizes of the corresponding fragments are highlighted in bold in **A**. DNA size markers were run in the right lane.

sized polypeptides (Chang et al., 1989; Meinnel and Blanquet, 1994). It is found in most bacterial species yet is absent from mammalian cells, making it an attractive target for antibiotic discovery. We have identified, using biochemical assays, a number of hydroxamic acid inhibitors of the *S. aureus* Pdf1 enzyme activity. One of these inhibitors, SB220334, an actinonin-like compound (Chen et al., 2000), has an IC_{50} of $\sim$0.1 μM against the purified enzyme, and was found to exhibit antibacterial activity against *S. aureus* (MIC, 8 μg/ml). In order to examine whether the cellular antibacterial activity of this compound was due to its direct action on the *S. aureus* enzyme, we adapted a strategy similar to that described earlier for *metRS*. We created a strain,

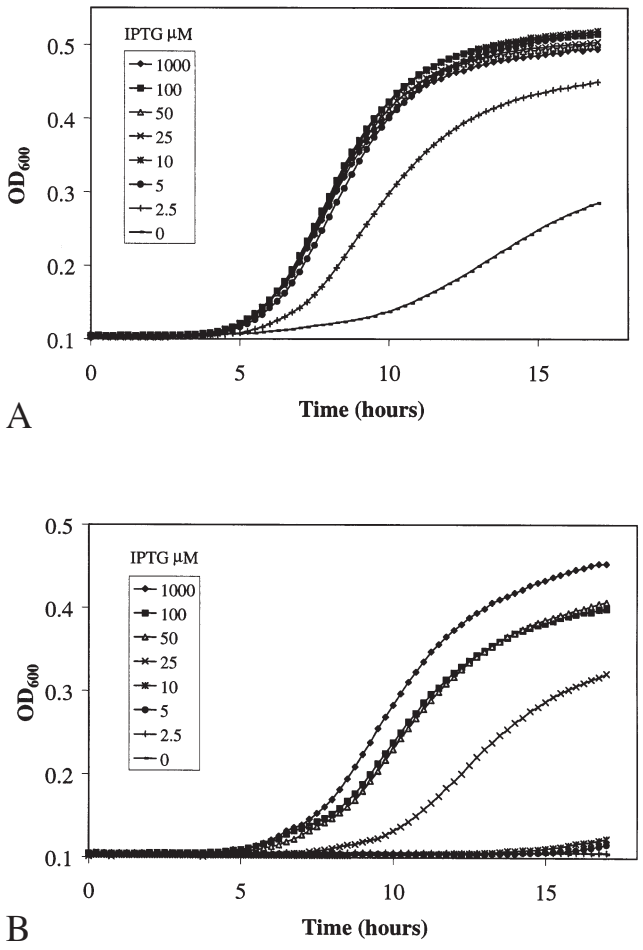


Fig. 5. Growth dependence on IPTG for *S. aureus* strains with Spac-regulated *metRS* gene. Growth curves were collected as described in Section 2.7. (**A**) Growth curves of FFmrs1 in TSB containing erythromycin and varying concentrations of IPTG. (**B**) Growth curves of FFmrs1(pFF40) in TSB containing erythromycin, kanamycin and varying concentrations of IPTG.

called FFdef1, in which the P_{spac} promoter is inserted immediately upstream of the *def1* gene encoding Pdf1 (GenBank accession No. AY007227). The insertion was confirmed by PCR using several diagnostic sets of primers and the induction of Pdf1 in strain FFdef1 was also confirmed by Western immunoblotting (data not shown). In addition, to ensure sufficient repressor levels, the LacI-expressing plasmid pFF40 was introduced into strain FFdef1. The cell growth of the resulting strain showed complete dependence on induction (data not shown), confirming the essentiality of Pdf1 for *S. aureus* viability.

We next examined the antimicrobial activity of compound SB220334 against strain FFdef1(pFF40) in media containing varying concentrations of inducer. The results (Table 1) demonstrate a clear correlation between the MIC values and the levels of induction. The MIC values were 8 μg/ml when fully induced (400 μM IPTG), and shifted dramatically in a dose-dependent manner down to 0.06 μg/ml at the lowest induction level (6 μM IPTG) that still supported cell growth. This represents an MIC shift of ≥100-fold. In contrast, the MIC values for a control antibiotic not targeting Pdf1 (mupirocin) were not altered by induction (Table 1). Therefore, the antibacterial activity of SB220334 appears to be due to its inhibition of the Pdf1 target enzyme.

Table 1
Antimicrobial activities (MIC values) of SB220334 and mupirocin against *S. aureus* strain FFdef1(pFF40) with *def1* expression under the control of Spac[a]

IPTG (μM)	400	200	100	50	25	12.5	6.3
SB220334 (μg/ml)	8	4	2	0.5	0.25	0.125	0.06
Mupirocin (μg/ml)	0.06	0.06	0.03	0.06	0.03	0.03	0.06

[a] MIC was determined by the broth microdilution method in 96-well microtiter plates with Mueller–Hinton broth according to NCCLS recommended guidelines (NCCLS, 1997). The MIC was the lowest concentration of drug that showed no visible growth after incubation at 35°C for 18–24 h, with a starting inoculum of 1×10^6 colony forming units (CFU)/ml.

4. Conclusions

In this report, we demonstrated that three regulated promoter systems, which were previously developed in *Bacillus*, all function and exhibit titratable induction in *S. aureus*. Together, they provide physiologically relevant protein expression levels of over three orders of magnitude.

The chromosomally-integrated Spac system, in combination with the LacI-expressing plasmid pFF40, provides an inducible, titratable and well-regulated system for testing the requirement of specific gene products for cell viability and creating conditional lethal phenotypes in *S. aureus*. In addition, strains with titratable gene products are useful for linking the antibacterial activity of an antibiotic with the proposed target mechanism. This information is extremely valuable for driving antibiotic drug discovery efforts against relevant pathogens.

Acknowledgements

This work was supported by a research grant from the Defense Advanced Research Projects Agency (DARPA) (N65236-97-1-5810). The content of this publication does not necessarily reflect the position or the policy of the Government, and no official endorsement should be inferred.

We thank Dr. Simon Foster for plasmids pMUTIN4 and pSMUTery, Dr. Wolfgang Hillen for plasmid pWH353, Dr. Olaf Schneewind for strain OS2, and Dr. Wolfgang Schumann for plasmid pX. We thank Tong

Li, Shawn Shearn, and the SmithKline Beecham Microbiology sequencing group for technical assistance. We also thank Drs. Martin Burnham, Wolfgang Ebel, David Holmes, Daniel Gentry, Michael Gywnn and Howard Kallender for helpful discussions and suggestions.

References

Apfel, C.M., Takacs, S., Fountoulakis, M., Stieger, M., Keck, W., 1999. Use of genomics to identify bacterial undecaprenyl pyrophosphate synthetase: cloning, expression, and characterization of the essential *uppS* gene. J. Bacteriol. 181, 483–492.

Bruckner, R., 1992. A series of shuttle vectors for *Bacillus subtilis* and *Escherichia coli*. Gene 122, 187–192.

Chan, P.F., Foster, S.J., 1998. The role of environmental factors in the regulation of virulence-determinant expression in *Staphylococcus aureus* 8325-4. Microbiology 144, 2469–2479.

Chang, S.Y.P., McGary, E.C., Chang, S., 1989. Methionine aminopeptidase gene of *Escherichia coli* is essential for cell growth. J. Bacteriol. 171, 4070–4072.

Chen, D.Z., Petel, D.V., Hackbarth, C.J., Wang, W., Dreyer, G., Young, D.C., Margolis, P.S., Wu, C., Ni, Z.J.T.J., White, R.J., Yuan, Z., 2000. Actinonin, a naturally occurring antibacterial agent, is a potent deformylase inhibitor. Biochemistry 39, 1256–1262.

Eichenbaum, Z., Federle, M.J., Marra, D., Devos, W.M., Kuipers, O.P., Kleerebezem, M., Scott, J.R., 1998. Use of the *lactococcal nisa* promoter to regulate gene expression in gram-positive bacteria — comparison of induction level and promoter strength. Appl. Environ. Microbiol. 64, 2763–2769.

Geissendorfer, M., Hillen, W., 1990. Regulated expression of heterologous genes in *Bacillus subtilis* using the Tn10 encoded tet regulatory elements. Appl. Microbiol. Biotechnol. 33, 657–663.

Gregory, P.D., Lewis, R.A., Curnock, S.P., Dyke, K.G., 1997. Studies of the repressor (BlaI) of beta-lactamase synthesis in *Staphylococcus aureus*. Mol. Microbiol. 24, 1025–1037.

Guzman, L.M., Belin, D., Carson, M.J., Beckwith, J., 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. J. Bacteriol. 177, 4121–4130.

Heid, C.A., Stevens, J., Livak, K.J., Williams, P.M., 1996. Real time quantitative PCR. Genome Res. 6, 986–994.

Holland, P.M., Abramson, R.D., Watson, R., Gelfand, D.H., 1991. Detection of specific polymerase chain reaction product by utilizing the 5′–3′ exonuclease activity of *Thermus aquaticus* DNA polymerase. Proc. Natl. Acad. Sci. USA 88, 7276–7280.

Iordanescu, S., 1993. Characterization of the *Staphylococcus aureus* chromosomal gene *pcrA*, identified by mutations affecting plasmid pT181 replication. Mol. Gen. Genet. 241, 185–192.

Ji, G., Silver, S., 1992. Regulation and expression of the arsenic resistance operon from *Staphylococcus aureus* plasmid pI258. J. Bacteriol. 174, 3684–3694.

Ji, Y., Marra, A., Rosenberg, M., Woodnutt, G., 1999. Regulated antisense RNA eliminates α-toxin virulence in *Staphylococcus aureus* infection. J. Bacteriol. 181, 6585–6590.

Kim, L., Mogk, A., Schumann, W., 1996. A xylose-inducible *Bacillus subtilis* integration vector and its application. Gene 181, 71–76.

Kraus, A., Iandolo, J.J., 1990. High-frequency transformation of *Staphylococcus aureus* by electroporation. Curr. Microbiol. 21, 373–376.

Kreiswirth, B.N., Lofdahl, S., Betley, M.J., O'Reilly, M., Schlievert, P.M., Bergdoll, M.S., Novick, R.P., 1983. The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. Nature 305, 709–712.

Meinnel, T., Blanquet, S., 1994. Characterization of the *Thermus thermophilus* locus encoding peptide deformylase and methionyl-tRNAfmet formyltransferase. J. Bacteriol. 176, 7387–7390.

NCCLS, 1997. Approved Standard M7-A4. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. National Committee for Clinical Laboratory Standards, Villanova, PA.

Nicholson, W.L., Setlow, P., 1990. Sporulation, germination and outgrowth. In: Molecular Biological Methods for *Bacillus*. Wiley, Chichester, UK, pp. 391–429.

Novick, R.P., 1991. Genetic systems in *Staphylococci*. Methods Enzymol. 204, 587–636.

Oskouian, B., Stewart, G.C., 1987. Cloning and characterization of the repressor gene of the *Staphylococcus aureus* lactose operon. J. Bacteriol. 169, 5459–5465.

Petit, M.A., Dervyn, E., Rose, M., Entian, K.D., McGovern, S., Ehrlich, S.D., Bruand, C., 1998. PcrA is an essential DNA helicase of *Bacillus subtilis* fulfilling functions both in repair and rolling-circle replication. Mol. Microbiol. 29, 261–273.

Rohrer, S., Ehlert, K., Tschierske, M., Labischinski, H., Berger-Bachi, B., 1999. The essential *Staphylococcus aureus* gene *fmhB* is involved in the first step of peptidoglycan pentaglycine interpeptide formation. Proc. Natl. Acad. Sci. USA 96, 9351–9356.

Schneewind, O., Model, P., Fischetti, V.A., 1992. Sorting of protein A to the staphylococcal cell wall. Cell 70, 267–281.

Stieger, M., Wohlgensinger, B., Kamber, M., Rolf, L., Keck, W., 1999. Integrational plasmids for the tetracycline-regulated expression of genes in *Streptococcus pneumoniae*. Gene 226, 243–251.

Vagner, V., Dervyn, E., Ehrlich, S.D., 1998. A vector for systematic gene inactivation in *Bacillus subtilis*. Microbiology 144, 3097–3104.

Yansura, D.G., Henner, D.J., 1984. Use of the *Escherichia coli lac* repressor and operator to control gene expression in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA 81, 439–443.